

Section of Experimental Medicine and Therapeutics

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Studies of Folate Deficiency in Man

Much of the interest in a hæmopoietic factor in the liver other than vitamin B₁₂ developed in large measure from the work of Lucy Wills (Wills 1932, Wills *et al.* 1937). She showed in a series of papers that macrocytic anæmia occurring among Hindu women in Bombay responded to injections of 'crude' but not 'refined' liver extract. As Girdwood (1960) and others have indicated, the unknown 'Wills factor' was undoubtedly folate, and folate deficiency and megaloblastic anæmia have been extensively studied in Britain. With no lack of appreciation for the many other British workers who provided inspiration for some of our studies, I would mention Ungley (1950, 1957), Witts (1961), Girdwood (1960) and Mollin (1960) as foci of a great many studies which have stimulated our imagination. We have reviewed many of these studies elsewhere (Herbert 1959), and shall here deal mainly with our own studies in this field.

Development of Serum Folate Assay with *Lactobacillus casei*

The problem of assessing folate deficiency in man was investigated in 1958 and 1959 with Herman Baker who had just given up an assay for folic acid in blood because *Bacillus coagulans*, the organism used, had mutated to a point where it was no longer folate dependent (Baker *et al.* 1959). The serum folate level as measured by *L. casei*, the organism used by Usdin *et al.* (1956) to study whole blood folates, proved to be a sensitive index of folate status in man (Herbert *et al.* 1959, Baker *et al.* 1959, Herbert *et al.* 1960). Many workers have confirmed this (Cooper & Lowenstein 1961, Waters & Mollin 1961, Hansen &

Weinfeld 1962, Izak *et al.* 1961, Davis & Kelly 1962, Arakawa *et al.* 1963, Lindenbaum & Klipstein 1963, Metz 1963, Cooke *et al.* 1963, Layrisse, personal communication).

For the past several years we have been using the 'aseptic addition method' (Herbert 1961*b*, Herbert & Zalusky 1962*a*) in which flasks containing 5 ml of double-strength medium, 4 ml of de-ionized water and 1 ml of pH 6.1 phosphate buffer to which 10 mg ascorbic acid was freshly added are autoclaved. To one flask is added 0.1 ml serum; to another 0.2 ml serum; strict asepsis is unnecessary in collecting or transferring the serum. A drop of *L. casei* is then added to each flask and incubation begun.

This method has the advantages of: (1) Halving the manipulations involved in the assay by deleting the step involving autoclaving of serum in buffer. (2) Allowing use of 1 ml of 1 g% ascorbate, which better preserves serum folates (not more than 350 mg% can be used before autoclaving in the 'standard method', described in 1959 by Baker *et al.* which most other workers now use, because greater amounts prevent precipitation of protein when serum is autoclaved). (3) Requiring only 0.3 ml serum (0.1 ml for one sample; 0.2 ml for its duplicate), an especially valuable point when children are studied.

The validity of the assay is unaffected by various minor changes in assay method or medium, as long as contamination of glassware and ingredients by folate is excluded (the commercial grade ingredients, especially casein and glucose, from many drug firms are contaminated with sufficient folate to invalidate the assay). The medium we use (Baker *et al.* 1959) is almost identical to that of Jukes (1955), but contains twice as much para-aminobenzoic acid. Waters & Mollin (1961) got results essentially identical with ours by reproducing our assay medium except that they used enzymatically hydrolysed casein instead of acid hydrolysed casein and tryptophan, a variation which Usdin *et al.* (1956) had considered less desirable for a 'dry mix' but equally good for a freshly prepared medium.

Our assay medium is commercially available as a 'dry mix' (just add water and stir) from Baltimore Biological Laboratories (Baltimore, Maryland, USA) and a very similar one from Difco Laboratories (Detroit, Michigan, USA). These 'dry mixes' should

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be refrigerated to avoid decomposition. They give identical results in our hands.

We routinely collect serum from fasting subjects, because we found that ingestion of chicken or beef liver produced sharp rises in serum *L. casei* (but not *Str. faecalis*) folate levels. For example, after a breakfast of chicken livers, one normal subject with a baseline *L. casei* folate level of 10 ng/ml within 0.5 h reached 20 ng/ml and climbed in another 0.5 h to values of 50–80 ng/ml sustained for four hours. No other food yet studied has shown this striking effect which indicates absorbability of liver folate so great as to be approached only by folic acid itself. It was the striking effect of liver folate which caused us to introduce it into Fig 6 (Herbert & Zalusky 1962a).

It should be noted here that the abscissa of Fig 1 of our paper on assay and nature of serum folate activity (Herbert & Zalusky 1961b) should have read 'Folic acid ($m\mu g/ml$)/100' since the actual folic acid concentration is 1/100 of the abscissa figure, as was made clear in Table 1 of the same paper. The unfortunate deletion of '/100' led several workers (Waters & Mollin 1961) to believe that we studied the effect of ascorbate on *L. casei* growth using larger concentrations of folic acid than are used in the assay, and more ascorbic acid, whereas in fact we used the same concentration of folic acid and autoclaved ascorbate-phosphate buffer as in the serum assays.

Again, it is emphasized that variations in methodology are generally unimportant. The important factors are: (1) Cleanliness to avoid contamination with folate (contamination with micro-organisms is rarely a problem because *L. casei* grows so fast and makes so much lactic acid). (2) Refrigeration of media to avoid deterioration. (3) Preservation of serum against oxidative destruction, which we will now take up in more detail.

Lability of Serum L. casei Folate

Ascorbate or other reducing agents such as 2-mercaptoethanol preserve the *L. casei* folate activity in the serum which, unlike the small amount of stable *Str. faecalis* activity, is highly labile and needs protection against oxidative destruction (Herbert 1960, 1961b). The lability of serum *L. casei* folate and its protection by ascorbate has been confirmed by others. Waters & Mollin (1961) initially recommended use of less ascorbate (100 mg%) to protect serum folate than the 150 mg% we found desirable in the 'standard method' (Herbert 1960, 1961b). More recently, they have confirmed the advisability of increasing the concentration of ascorbate above 100 mg% (Waters & Mollin 1963).

In our experience the addition of ascorbate to serum before freezing (Waters & Mollin 1961) at $-20^{\circ}C$ is unnecessary if the samples will be assayed within two years (Herbert 1962b, Davis & Kelly 1962). However, if storage before assay will exceed two years, addition of a reducing agent is desirable. Incidentally, the addition of ascorbate partially destroys vitamin B₁₂ (Hutchins *et al.* 1956), and too much ascorbate destroys folate (Scheidlin & Griffith 1951).

The Nature of Serum L. casei Folate

The simple finding that the intermediate of methionine biosynthesis of Larrabee and Buchanan grew on *L. casei* (Herbert, quoted by Larrabee *et al.* 1961) suggested three important possibilities, all of which seem to have proved true: (1) That the intermediate was the same as Donaldson & Keresztesy's 'prefolic A' (1959) (Keresztesy had written to us in January 1961 that 'prefolic A' grew on *L. casei*). (2) That both the intermediate and 'prefolic A' may be the same as the major folate in human serum. (3) That there existed a class of folate monoglutamates, such as 5-CH₃-folate-H₄, which grew on *L. casei* but not on *Str. faecalis* (Herbert *et al.* 1962).

Concurrently with the studies of Larrabee *et al.* (1961, 1963), which defined the intermediate as being 5-CH₃-folate-H₄, serum folate was concentrated on and eluted from charcoal, and shown by chromatography in four separate solvent systems to be 5-CH₃-folate-H₄ (or 5-CH₃-folate-H₂, which migrates identically) (Herbert *et al.* 1962) and not to be 5-CH₃-folate-tri-glutamate-H₄ which was synthesized to help rule out the possibility (Herbert 1961b) that the main serum folate might be a triglutamate. In this connexion, it is *still* possible that the main folates in man may be conjugated since we have not ruled out the possible action on serum folate of natural conjugases in serum before chromatography (Noronha & Aboobaker 1963).

Production of Pure Nutritional Folate Deficiency in Man

With the possible exception of one study in which baseline folate stores were not determined (Knowles *et al.* 1961, Fleming *et al.* 1963), pure nutritional folate deficiency had not been produced in normal man until 1962 (Herbert 1962c). In this study, a healthy 35-year-old physician with normal baseline erythrocyte folate stores developed folate deficiency megaloblastic anaemia after four and a half months on a diet from which the folate content had been extracted by large quantities of boiling water (Herbert 1963a). In brook trout anaemia develops after only nine weeks of folate deprivation (Phillips 1963).

It takes approximately 120 days, or the life span of a red cell, for the normal-folate-containing red cells to be completely replaced by folate-deficient red cells, suggesting that mature erythrocytes do not release their folate until they die (Fig 1). Other evidence (Herbert & Zalusky 1962a) indicates that folate enters only young erythrocytes and/or their precursors.

Fig 2 shows the sequence of events in this instance of developing folate deficiency. This sequence parallels in many ways the sequence of events in development of deficiency of ascorbic

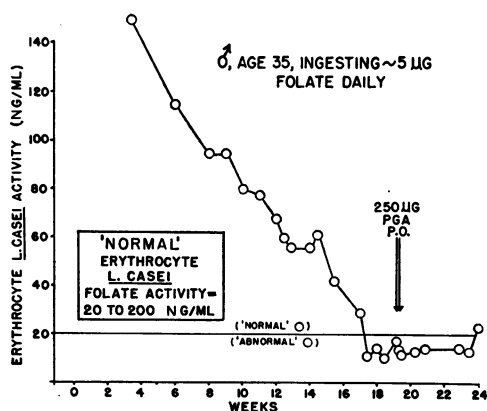


Fig 1 Erythrocyte folate activity in developing experimental nutritional folate deficiency (Reproduced from Herbert, 1962c, by kind permission)

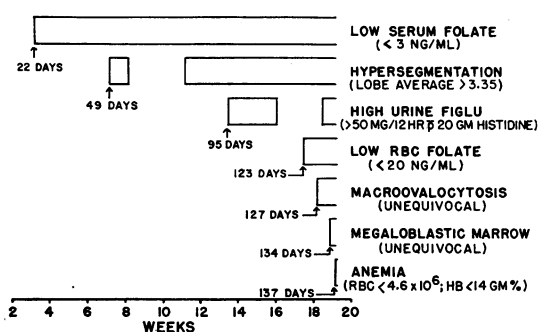


Fig 2 Experimental nutritional folate deficiency in man: biochemical and hematological sequence of events (Modified from Herbert, 1962c, by kind permission)

acid, another water-soluble vitamin (Crandon *et al.* 1940, Krebs *et al.* 1948). As do ascorbate levels in developing ascorbate deficiency, the serum folate level becomes low within several weeks after cessation of ingestion of folate, and only several months later does the level of folate in formed elements of the blood become subnormal. The rapidity with which folate stores are exhausted when folate intake (or absorption) ceases may explain the 'acute folic acid deficiency of unknown aetiology' of the patients of Cooke *et al.* (1963), who may have started with low folate stores. The first indication of megaloblastic change in the bone marrow was seen in the specimen obtained twenty-three days after the onset of folate deprivation (Herbert 1964) although, as noted in Fig 2, gross unequivocal megaloblastosis does not appear until much later.

Although various tests for intestinal malabsorption were all still normal when megaloblastosis appeared, and we described the jejunal biopsy as 'essentially normal', it did show two possibly questionable phenomena: (1) Infiltration

of the lamina propria by lymphocytes. (2) Occasional large nuclei in the columnar cells. More pronounced similar phenomena were previously noted in jejunal biopsies in sprue (Butterworth & Perez-Santiago 1958), and Ten Thije (1963) reported that columnar cell megaloblastic nuclei in jejunal biopsies of 2 patients with sprue and megaloblastic anaemia returned to normal after folic acid therapy. Sheehy (1964) noted that American soldiers in Puerto Rico who were developing sprue had megaloblast-like nuclei in the columnar cells of their jejunal biopsies at a time when they had peripheral blood hypersegmentation and macro-ovalocytes but no anaemia.

Jejunal biopsies of the experimental folate deficiency subject were taken on day 123 of folate deprivation, just before the development of anaemia, and again on December 5, 1963, twenty-one months after resuming a normal diet. These two biopsies show similar infiltration of lamina propria and occasional large nuclei in columnar cells, which confirms our initial impression that the jejunal biopsy was normal at a time when the bone marrow was clearly abnormal. This may suggest either that the jejunal mucosa is unaffected by pure folate deficiency or, more probably, that it keeps the tiny (~5 µg daily) amount of ingested folate for its own needs rather than passing it on to the deprived marrow. Other possibilities are that the epithelium of the gastrointestinal tract has a selective advantage over haemopoietic cells in securing available folate, or requires less folate to sustain normality.

The 'Lobe Average'

To facilitate charting of the degree of hypersegmentation, we have for several years used the 'lobe average', i.e. the total number of nuclear lobes in 100 neutrophils divided by 100 (Herbert 1962c). This single figure is simpler to work with than the 'Arneth count' (Hynek 1909, Arneth 1945, Wintrobe 1939, Herbert 1959) which is a listing of the percentage of cells with 1, 2, 3, 4 and 5 or more nuclear lobes among 100 neutrophils. In 1909 Hynek had described an almost identical simplification when he reported use of a lobe total (i.e. the number of segments in 100 neutrophils). The 'lobe average' is now being used in other laboratories in the United States, and also in London (Ardeman *et al.* 1963). We feel that it will prove as useful as the MCV (mean corpuscular volume, Wintrobe 1961) in assessing megaloblastic anaemia, especially since the 'lobe average' becomes elevated before the MCV. It should be noted that not all individuals agree on what constitutes separation between lobes, and thus the 'normal' lobe average must be separately determined by each laboratory (in our laboratory,

it is 3.17 ± 0.25). The crucial factor is that the hypersegmented neutrophil seems to contain more nuclear material than normal. In our unpublished study, the area occupied by 100 nuclei was 10% greater for neutrophils from a subject with megaloblastic anaemia than from a normal subject.

Incidentally, as Undritz (1952) noted, 'twinning deformities' should not be confused with 'hypersegmented neutrophils'. The former contain two nuclei in one cytoplasm, and are twice the size of normal (or hypersegmented) neutrophils. They occur rarely in normal blood and more frequently with megaloblastic anaemia or blood malignancies. Fig 3 shows a 'twinning deformity' next to a four-lobed neutrophil (blood smear obtained from subject of Figs 1 and 2, seventy-eight days after onset of folate deprivation). It must be remembered that an increased lobe average occurs for unknown reasons with chronic renal disease, despite normal serum vitamin B₁₂ and folate levels (Herbert, unpublished), and also with congenital hypersegmentation of the neutrophil nucleus (Undritz 1958, Barbier 1958). In this entity, which occurs in approximately 1% of normal adults in Boston, the hypersegmented neutrophils are oval (as in megaloblastic anaemia), but serum vitamin B₁₂ and folate levels are normal (Herbert, Kapff & Jaskiel, unpublished).

Therapeutic Tests in Suspected Folate Deficiency

The principles involved in the use of reticulocyte responses in therapeutic tests of erythropoietic substances were laid down in a classical paper by Minot & Castle in 1935 and have been recently reviewed (Herbert 1963b). Although it was known that small doses of folic acid might produce therapeutic response only in patients with folate deficiency, and not in patients with vitamin B₁₂ deficiency (Ungley 1957, Chanarin *et al.* 1958), it was not until the study of Marshall & Jandl (1960) that a paper was devoted solely to the subject of differential diagnosis of folate deficiency from vitamin B₁₂ deficiency by use of 'small' (0.4 mg i.m. daily) doses of folic acid. One of the 3 patients of Marshall & Jandl with vitamin B₁₂ deficiency showed a slight reticulocyte response to this dose of folic acid, and we subsequently noted moderate reticulocyte responses in 2 patients with vitamin B₁₂ deficiency given this dose (Zalusky & Herbert 1961, Herbert 1962b, 1963b). The use of 0.1 mg folic acid daily in therapeutic tests is therefore recommended (Zalusky & Herbert 1961, Chosy *et al.* 1962, Hansen & Weinfeld 1962).

Sheehy *et al.* (1961) obtained haematological responses to 25 µg of folic acid orally daily in all patients with folate deficiency in nontropical sprue, when not complicated by vitamin B₁₂

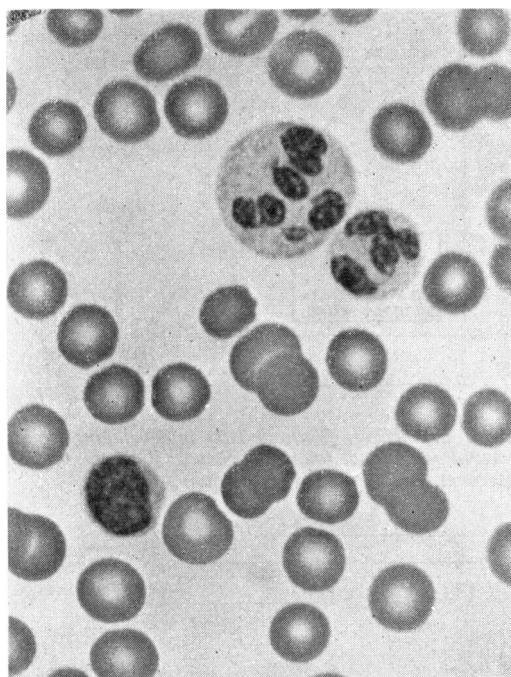


Fig 3 'Twinning deformity' next to four-lobed neutrophil (peripheral blood of normal man after seventy-eight days of deprivation of dietary folate)

deficiency, suggesting that therapeutic tests with folic acid may be conducted as successfully orally as subsequently done with 25 µg folic acid daily intramuscularly (Zalusky & Herbert, unpublished). However, when only 25 µg of folic acid is superimposed on an experimental diet essentially devoid of folate, there may be no therapeutic response (Sullivan & Herbert 1963). The situation here is unlike that of the patients of Sheehy *et al.* who received their 25 µg of folic acid orally daily superimposed on a diet containing from 1,000 to 1,500 µg of total folate (a small fraction of which may have been available for absorption, thereby augmenting the effect of the administered folic acid).

Study of Minimal Daily Adult Folate Requirement

Since the study of experimental nutritional folate deficiency in man had indicated that the first abnormality was a fall in the serum folate level, it became easy to look into the minimal daily folate requirement, starting from the premise that this requirement could be equated with the amount of folic acid necessary to sustain a normal serum folate level. Three healthy young adult female medical research technicians were placed on the essentially no-folate diet with supplements of 25, 50 and 100 µg of pteroylglutamic (folic) acid daily. A significant fall in serum folate level occurred in the subject supplemented with 25 µg

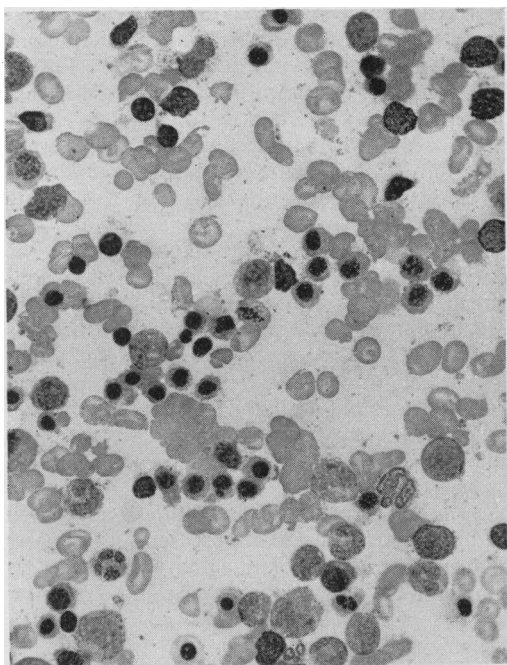


Fig 4 Subject with borderline marrow morphology, due to poor diet in association with alcoholism producing folate deficiency. (Sullivan & Herbert, unpublished.)

of folic acid daily but not in the subjects supplemented with 50 or 100 μg daily (Herbert 1962a). This indicated a minimal daily adult folate requirement in the range of 50 μg of folic acid, which agrees with the findings of Fleming *et al.* (1963).

Other studies (Sullivan & Herbert 1963) also tend to support the figure of approximately 50 μg of folic acid per day as in the range of the minimal daily folate requirement. In these studies, a 61-year-old woman was sustained on the essentially no-folate diet from May 1962 until the present, and her severe folate-deficiency megaloblastic anaemia was shown to be correctable by 75 μg of folic acid daily, indicating that this quantity improved her status and that less would sustain it. Also supporting the 50 μg figure is a study in which the blood of a subject with megaloblastic anaemia returned toward normal when a diet totally devoid of folate was given, supplemented with 50 μg of folic acid daily (Zalusky & Herbert 1961).

Production of Overt Megaloblastosis Within Two Weeks

The last-mentioned patient, with borderline folate deficiency and a marrow not clearly megaloblastic, developed gross overt megaloblastosis within two weeks when he was given a diet devoid of folate (Zalusky & Herbert 1961). This

same phenomenon, rapid development of overt megaloblastosis, has been repeatedly induced within ten days by feeding alcohol to subjects with folate deficiency and borderline marrow morphology (Sullivan & Herbert 1963) (Figs 4 and 5). In fact, there is now strong evidence that alcohol suppresses haemopoiesis by interfering with folate metabolism (Sullivan & Herbert 1963) as well as evidence that folate deficiency is usual in alcoholics (Herbert *et al.* 1963).

*Selective Concentration of *L. casei* Folate in Spinal Fluid*

In comparing serum and spinal fluid levels of *L. casei* folate activity, it was found that *L. casei* folate selectively concentrated in spinal fluid (Herbert & Zalusky 1961b). This raises the possibility that an antimetabolite of 5-CH₃-folate-H₄ may be more effective therapy for malignancy affecting the nervous system than antifolates such as aminopterin or methotrexate since both of these may be selectively excluded from the spinal fluid (Shaw *et al.* 1960, Delmonte & Jukes 1962). Furthermore, preliminary studies suggest that methotrexate has a half time of less than thirty minutes in the spinal fluid and may be actively transported out of the spinal fluid (W W Oppelt & D P Rall, personal communication).

We are currently studying why neurological damage more severe than sleeplessness, forgetfulness and irritability (Herbert 1962c) does not

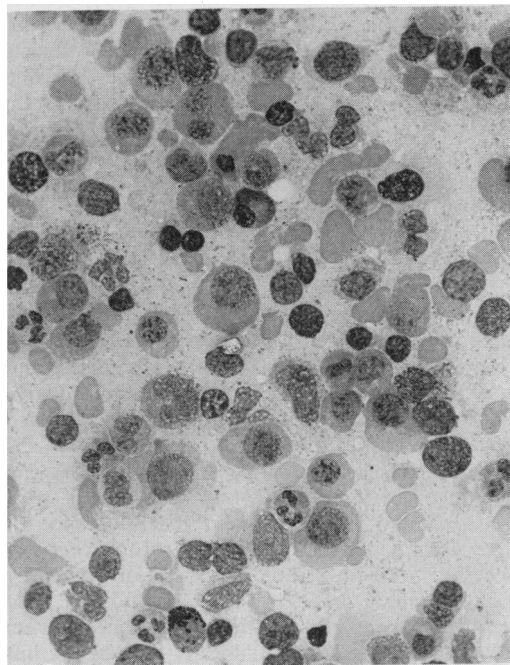


Fig 5 Same subject as Fig 4, ten days later, after 15 oz whisky daily. (Sullivan & Herbert, unpublished.)

develop in folate deficiency. It may be relevant that the selective concentration of *L. casei* folate activity in the spinal fluid operates even during developing folate deficiency, and may (or may not) reflect an ability of the nervous system to secure folate constantly as needed. Of 11 subjects with serum *L. casei* folate activity between 4.2 and 6.3 ng/ml, all had normal spinal fluid *L. casei* folate activities (19 to 39 ng/ml), suggesting that although the serum level may have been falling, the spinal fluid level was not. Three patients with folate deficiency megaloblastic anaemia had serum *L. casei* folate levels of 2.2, 1.9 and <1 ng/ml and spinal fluid levels of 7, 13 and 7.9 ng/ml respectively, which, while below normal, were still at least three times the serum level.

Secondary Folate Deficiency due to Vitamin B₁₂ Deficiency

The concept that much of the megaloblastic anaemia of vitamin B₁₂ deficiency may be the result of inadequate folate utilization due to the vitamin B₁₂ deficiency has been entertained by many workers for many years (Herbert 1959, Vilter *et al.* 1963, Waters & Mollin 1963). Among the first 10 patients with pernicious anaemia whose serum *L. casei* folate activity was determined, one had an inordinately high level (Herbert *et al.* 1960). Further study showed the incidence of such high *L. casei* folate activity in the serum of 100 of our patients with vitamin B₁₂ deficiency to be 17% rather than 10% (Herbert & Zalusky 1962a) and demonstrated normal serum folate in many vitamin-B₁₂-deficient subjects despite protracted reduced folate ingestion due to anorexia which should have made serum folate levels subnormal (Herbert & Zalusky 1961c). At least two types of folate deficiency appeared to result from vitamin B₁₂ deficiency: (1) Inadequate folate utilization due to lack of vitamin B₁₂. (2) Inadequate folate ingestion due to anorexia resulting from vitamin B₁₂ deficiency.

Chanarin *et al.* (1958) reported that *Str. faecalis* activity disappeared rapidly from the serum of subjects with severe pernicious anaemia after intravenous injection of folic acid, and interpreted this to mean 'an increase in the degree of "unsaturation" of the tissues for folic acid, and that these tissues are, therefore, depleted of the vitamin'. Reproducing their studies, but assaying not only with *Str. faecalis* but also with *L. casei*, we suggested that the rapid disappearance of *Str. faecalis* activity from the serum was *not* necessarily due to rapid removal of folate by folate-depleted tissue, but rather could be due in significant measure to rapid conversion of serum folate to a form, unavailable to *Str. faecalis* but available to *L. casei*, which accumulated in the serum (Herbert 1961a, Herbert & Zalusky 1961a).

These data, plus the fact that formiminoglutamate excretion in the urine of one-third of pernicious anaemia patients was inordinately high (Herbert 1959, Herbert & Zalusky 1961d, 1962b) delineated further the thesis (Herbert & Zalusky 1961c) that an *L. casei*-active folate accumulated in the serum of vitamin-B₁₂-deficient subjects, and that vitamin B₁₂ is necessary for utilization of this folate (Herbert & Zalusky 1961a).

Accumulation of *L. casei*-active folate in the serum in vitamin B₁₂ deficiency has been confirmed by Waters & Mollin (1961, 1963), Mollin *et al.* (1962) who used tritiated folic acid, Stevens *et al.* (1962), Hansen & Weinfeld (1962) and Cooper & Lowenstein (1963). The last two groups also found a relatively low *L. casei* folate activity in the erythrocytes of some patients with vitamin B₁₂ deficiency, suggesting that vitamin B₁₂ plays a role in the transport of folate into maturing erythrocytes. This finding is supported by our report of an almost ten-fold greater erythrocyte *L. casei* folate activity of 'young' as compared to 'old' erythrocytes eight days after the start of vitamin B₁₂ therapy for vitamin B₁₂ deficiency.¹

As we stated then (Herbert & Zalusky 1962a): 'The phenomenon (sharp fall in serum *L. casei* folate on therapy with vitamin B₁₂) may be due to release of the block in utilization of *L. casei* folic acid activity caused by lack of vitamin B₁₂ with subsequent rapid utilization in haemopoiesis, and may be similar to the fall in serum iron during therapy.' However, the proportion of the erythrocyte folate rise due to relatively greater influx and low efflux of reticulocyte folate is uncertain. It is possible that vitamin B₁₂ may play a role in transport of folate into all cells and/or retention of folate by cells as well as in utilization by cells. Liver *L. casei* and *Leuconostoc citrovorum* folate activities have been reported to fall to very low levels when sheep become severely vitamin-B₁₂-deficient (Dawbarn *et al.* 1958). The studies of Fischer *et al.* (1963) on uptake of tritium-labelled methotrexate by human leukæmic leucocytes further suggest that folate uptake and retention by cells must be considered in any explanation of why a given cell has a given folate level. The nature of the folate must also be considered. For example, leucocytes take up much more folic

¹It is worth noting that just before starting therapy, the patient's serum *L. casei* folate level was 35 ng/ml and the erythrocyte level 11.4 ng/ml; but on the seventh day of therapy she had a serum level of 5.8 ng/ml and an erythrocyte level of 252 ng/ml. On both occasions her serum *Str. faecalis* folate level was <1 ng/ml; just before therapy her erythrocyte *Str. faecalis* folate level was 3.8 ng/ml and on the seventh day of therapy it was 10 ng/ml. However, Cox *et al.* (1960) reported that whole blood *Str. faecalis* folate levels rose only temporarily in patients responding haematologically to vitamin B₁₂, folic acid, iron and acute blood loss, and subsided when the haemoglobin rise subsided. This may suggest that the folate content of reticulocytes, however produced, is greater than that of mature erythrocytes.

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Studies on Urinary Formiminoglutamic Acid Excretion

In 1951 Bakerman *et al.* found that rats maintained on a folic-acid-deficient diet excreted an unstable form of glutamic acid in the urine, which was subsequently identified as formiminoglutamic acid. The early work on folic acid function had shown that the amino acid histidine contributed carbon-2 of the imidazole ring to the single-carbon unit pool and it did so in the following manner (Rabinowitz 1960): The main pathway of histidine catabolism is to urocanic acid which is converted to formiminoglutamic acid by opening of the imidazole ring. The next stage is the transfer of the formimino-($-\text{CH}=\text{NH}$) group to the folate coenzyme. If the function of the folate coenzyme is interfered with, formiminoglutamic acid (figlu) accumulates and may appear in the urine.